REMARKS/ARGUMENTS

With this response, claims 1-8, 14-17, 21-26, 35-38 and 51-58 are pending. Claims 9-13, 18-20, 27-34, and 39-41 are withdrawn. Claims 42-50 and 59-62 are cancelled. For convenience, the Examiner's rejections are addressed in the order presented in an November 1, 2005, Office Action.

I. Priority claim

According to the Office Action, Applicants have not complied with conditions for receiving the benefit of an earlier filed application, *i.e.*, allegedly, no reference to the earlier filed provisional application is made in the specification. In response, Applicants respectfully request the Examiner to review the paragraph beginning at page 1, line 4. The paragraph is also reproduced here: "The present application claims the benefit of U.S. Provisional Application No. 60/400,583, filed August 2, 2002, which is herein incorporated by reference in its entirety." Applicants believe that this statement is sufficient to comply with requirement for reference to the earlier filed provisional application. As this paragraph was present in the Application as filed, Applicants respectfully request that any objections to the priority claim be withdrawn.

Applicants have amended the paragraph beginning at page 1 line 7 to include reference to a related application filed on the same day as the present application. This paragraph was included to incorporate the related application by reference and for the convenience of the Examiner.

II. Rejections under 35 U.S.C. §103(a)

A. Introduction

Various combinations of claims are rejected for alleged obviousness in view of various combinations of references. Applicants respectfully traverse the rejections.

The Office Action has not established a case of *prima facie* obviousness. To establish a case of *prima facie* obviousness, the Examiner must meet three basic criteria:

> First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the references or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. *In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991). M.P.E.P. §§ 706.02(j) and 2143.

The references cited by the Examiner fail to provide a reasonable expectation of success in practicing the invention and fail to provide a motivation for the combination of the references. In addition, the references cited by the Examiner fail to provide all the elements of the rejected claims. Therefore, Applicants respectfully traverse the rejections.

B. Rejection of claims 1-8

Claims 1-8 are rejected as obvious over Markovic *et al.*, US Patent No. 5,358,855 in view of Weber *et al.*, US Patent No. 5,405,837, further in view of Chen *et al, Jap. J. Cancer* (1990); and Leoni *et al., J. Nat'l Cancer Inst.* (2000).

Claims 1-8 are directed to methods to treat cancer by administering to a subject a combination of an inhibitor of the inosine monophosphate dehydrogenase (IMPDH) enzyme and an agent that inhibits a cellular process regulated by GTP or ATP. The cited references (discussed below) do not provide all the elements of the claimed methods. Moreover, Examples 1 and 2 of the specification provide evidence that, at a minimum, the subgenus of methods encompassed by claims 2-8 have unexpected benefits, not known to those of skill until the filing of this application.

Markovic *et al.*, US Patent No. 5,358,855

Markovic *et al.* disclose a role for IMPDH inhibitors as cancer chemotherapeutics. Applicants agree with the Office Action at page 4, that Markovic *et al.* do not teach the administration of IMPDH inhibitors in combination with other cancer chemotherapeutics as a treatment for cancer.

Weber et al., US Patent No. 5,405,837

Weber *et al.* teach treatment of cancer by administering two compounds that inhibit activity of the IMPDH enzyme: tiazofurin and ribavirin. No other chemotherapeutic compounds are taught or suggested for administration with tiazofurin and the dosages are tailored to the patient by monitoring the GTP concentration and the activity of the IMPDH enzyme.

Chen *et al, Jap. J. Cancer* (1990)

Chen *et al.* disclose circumstances under which mizoribine, an IMPDH inhibitor, enhances the growth of tumor cells. The authors had the goal of developing a cost-effective version of the murine subrenal capsule assay (SRCA), in which foreign tumor cells are inserted into and allowed to grow in the renal capsule of an immune compromised mouse. Candidate drugs are administered to the mouse and their effect on growth of the tumor is analyzed. Since the tumor cells are foreign to the host mouse, the tumor cells are at risk of death caused by an immune response by the host mouse. Therefore, the first SRCA models used genetically immune compromised mice, *e.g.*, nude or SCID mice. Because genetically immune compromised mice are expensive to buy and to house, Chen *et al.* substituted wild type mice, but administered mizoribine to inactivate the immune system thereby allowing growth of the tumor cells. At low mizoribine doses, the immune system was not inactivated and the tumor cells did not grow. At high mizoribine levels, the host mouse immune system is inactivated and the growth of tumor cells actually increased. However, the authors also noted that the effective (high) mizoribine dose for immune system inactivation is toxic to the host mice.

Leoni *et al., J. Nat'l Cancer Inst.* (2000)

Leoni *et al.* disclose that indanocine inhibits α-tubulin polymerization and has antiproliferative activity against cell lines derived from human cancers, including multi-drug resistant cell lines.

The cited references, alone or together, do not teach or suggest all the elements of the claimed invention and do not provide a motivation for their combination to arrive at the claimed invention. According to the Office Action, Weber *et al.* teach generally treatment of cancer comprising administration of tiazofurin with another chemotherapeutic agent. This is incorrect. Tiazofurin is an IMPDH inhibitor. Weber *et al.* address a problem of toxic reaction or resistance to tiazofurin administration by administering a second IMPDH inhibitor, *i.e.* ribavirin,. The use of a second chemotherapeutic agent with a different mechanism of action, *e.g.*, an agent that inhibits a cellular process regulated by GTP, such as tubulin polymerization, de novo purine metabolism, activity of a GPCR protein, or that inhibits DNA replication is not disclosed or suggested by Weber *et al.* Weber *et al.* does not disclose any need for combining tiazofurin with non-IMPDH inhibitors and so does not provide motivation for combination of the cited references to arrive at, *e.g.*, an IMPDH inhibitor combined with an inhibitor of α-tubulin polymerization.

According to the Office Action, Chen *et al.* teaches that mizoribine, an IMPDH inhibitor, effectively reduces the size of tumors. However, as discussed above, Chen *et al.* actually disclose circumstances where administration of mizoribine inhibits the immune response, resulting in increased growth of tumor cells. Thus, Chen *et al.* fails to contribute required elements of the claims and motivation to combine the cited references.

The other cited references, Markovic *et al.* and Leoni *et al.*, fail to correct the deficiencies of Weber *et al.* and Chen *et al.*

The specification also discloses that treatment of cancer cells with an IMPDH inhibitor and an agent that inhibits α-tubulin polymerization unexpectedly increases cancer cell death. *See,* specification, *e.g.*, at page 23, line 4 through page 25, line 11. A specific example is found at Example 2, page 51, line 31 through page 52 line 11 and Fig. 2. The example shows the results of treating chronic lymphocytic leukemia (CLL) cells with either indanocine, mizoribine, or a combination of indanocine and mizoribine. Without any treatment 65% of the CLL cells were viable 24 hours after the start of the experiment. Treatment with 1 μM indanocine resulted in death of more than half of the surviving CLL cells (24% viability). Treatment with 1 μM or 10 μM indanocine resulted in only a negligible decrease in CLL cell viability (55% or 45%,

respectively). However, when indanocine and mizoribine (both concentrations) were administered to the CLL cells, the cell viability unexpectedly dropped to less than 10% of the surviving cells. These surprising results support the non-obviousness of the claimed methods.

C. Rejection of claims 14-17

Claims 14-17 are rejected as obvious over Markovic *et al.*, US Patent No. 5,358,855 in view of Weber *et al.*, US Patent No. 5,405,837, further in view of Chen *et al*, *Jap. J. Cancer* (1990); and Ucken *et al.*, *Blood* (1998).

Claims 14-17 depend from claim 1 and are directed to methods to treat cancer by administering to a subject a combination of an IMPDH inhibitor and a precursor or prodrug of 9-beta-D-arabinofuranosylguanine 5'-triphosphate (Ara-GTP). Markovic *et al.*, Weber *et al.*, and Chen *et al*, are discussed above. Ucken *et al.* is discussed below. The cited references do not provide all the elements of the claimed methods. Moreover, the specification at page 25, lines 13-32 discloses improved results in killing cancer cell with the combination of an IMPDH inhibitor and a precursor or prodrug of Ara-GTP.

Ucken *et al.*, *Blood* (1998)

According to the Office Action, Ucken *et al.* disclose that Ara-G is selectively cytotoxic for T-cell lines and T-lineage leukemic cells.

The cited references, alone or together, do not teach or suggest all the elements of the claimed invention and do not provide a motivation for their combination to arrive at the claimed invention. Applicants refer the Examiner to section IIB, above, for discussion of the deficiencies of Weber *et al.* and Chen *et al.*, neither of which contributes the required elements of the claims and motivation to combine the cited references. The other cited references, Markovic *et al.* and Ucken *et al.* fail to correct the deficiencies of Weber *et al.* and Chen *et al.*

At page 25, lines 13-32, the specification discloses treatment of cancer using an IMPDH inhibitor in combination with an Ara-G precursor or prodrug. Because administration of the IMPDH inhibitor should cause a decrease in cellular GTP, the cells should be more

susceptible to the inhibition of DNA replication caused by Ara-G. Therefore, administration of the combination of drugs should be more effective than expected based on the individual use of each drug.

D. Rejection of claims 21-26

Claims 21-26 are rejected as obvious over Markovic *et al.*, US Patent No. 5,358,855 in view of Weber *et al.*, US Patent No. 5,405,837, further in view of Chen *et al*, *Jap. J. Cancer* (1990); and Carrera *et al.*, US Patent No. 5,840,505.

Claims 21-26 depend from claim 1 and are directed to methods to treat cancer by administering a combination of an IMPDH inhibitor and an agent that inhibits and an inhibitor of the de novo pathway of purine biosynthesis. Markovic *et al.*, Weber *et al.*, and Chen *et al*, are discussed above. Carrera *et al.* is discussed below. The cited references do not provide all the elements of the claimed methods. Moreover, the specification at paged 26-27 discloses improved results in killing cancer cells with the combination of an IMPDH inhibitor and an inhibitor of the de novo pathway of purine biosynthesis. The improved results are exemplified at, *e.g.*, Example 4, pages 52-53.

Carrera *et al.*, *Blood* (1998)

According to the Office Action, Carrera *et al.* disclose that L-alanosine, an inhibitor of purine biosynthesis, can be used to treat cancer cells, including cancer cells that lack MTAse activity.

The cited references, alone or together, do not teach or suggest all the elements of the claimed invention and do not provide a motivation for their combination to arrive at the claimed invention. Applicants refer the Examiner to section IIB, above, for discussion of the deficiencies of Weber *et al.* and Chen *et al.*, neither of which contributes the required elements of the claims and motivation to combine the cited references. The other cited references, Markovic *et al.* and Carrera *et al.* fail to correct the deficiencies of Weber *et al.* and Chen *et al.*

Moreover, at page 26, line1 through page 27, line 22, the specification discloses treatment of cancer using an IMPDH inhibitor in combination with an inhibitor of de novo purine biosynthesis. A specific example is found at Example 4, page 52, line 24 through page 53 line 4 and Fig. 4. The example shows that for lung cancer cells (A569 cells) the IC_{50} and IC_{90} of L-alanosine unexpectedly decrease with increasing amounts of mizoribine-base. Without any mizoribine present, the IC_{50} of L-alanosine is 5 μM and the IC_{90} of L-alanosine is 20 μM. With 5 μM mizoribine present, the IC_{50} of L-alanosine drops to 0.5 μM and the IC_{90} drops to of L-alanosine is 9 μM. With 25 μM mizoribine present, the IC_{50} of L-alanosine is 0.25 μM and the IC_{90} of L-alanosine is 6 μM. With 50 μM mizoribine present, the IC_{50} of L-alanosine decreases to 0.15 μM and the IC_{90} of L-alanosine decreases to 4 μM. These surprising results support the non-obviousness of the claimed methods.

E. Rejection of claims 35-38

Claims 35-38 are rejected as obvious over Markovic *et al.*, US Patent No. 5,358,855 in view of Weber *et al.*, US Patent No. 5,405,837, further in view of Chen *et al*, *Jap. J. Cancer* (1990); and Weers *et al.*, US Patent Application No. 2003/0003057.

Claims 35-38 are directed to methods to treat cancer by administering to a subject a combination of an inhibitor of the IMPDH enzyme and an agent that inhibits a cellular process regulated by GTP that is an antagonist of a G-protein coupled receptor (GPCR). Markovic *et al.*, Weber *et al.*, and Chen *et al*, are discussed above. Weers *et al.* is discussed below. The cited references do not provide all the elements of the claimed methods. Moreover, the specification at pages 34-36 discloses improved results in killing cancer cells with the combination of an IMPDH inhibitor and a GPCR antagonist.

Weers *et al.*, *Blood* (1998)

According to the Office Action, Weers *et al.* disclose administration of inhaled leuprolide to treat a variety of conditions. Weers *et al.* does not suggest administration of leuprolide with another chemotherapeutic agent.

The cited references, alone or together, do not teach or suggest all the elements of the claimed invention and do not provide a motivation for their combination to arrive at the claimed invention. Applicants refer the Examiner to section IIB, above, for discussion of the deficiencies of Weber *et al.* and Chen *et al.*, neither of which contributes the required elements of the claims and motivation to combine the cited references. The other cited references, Markovic *et al.* and Weers *et al.* fail to correct the deficiencies of Weber *et al.* and Chen *et al.*

At page 34, line 4 through page 36, line 11, the specification discloses treatment of cancer using an IMPDH inhibitor in combination with a GPCR antagonist. GPCR activity is increased in some cancer cells. GPCR proteins act by regulating intracellular signal transduction pathways. Applicants present Exhibit A, Lee *et al.*, *Proc. Nat'l Acad. USA* 103:1828-1833 (2006), as evidence that IMPDH inhibitors can act synergistically with regulators of signal transduction pathways. Lee *et al.* demonstrate that ribavirin and mizoribine affect activity of the DNA-dependent protein kinase and that mizoribine enhances activation of intracellular protein kinases by a toll like receptor agonist. *See, e.g.*, page 1830 and Figures 4 and 7. (Figure 7 is from supplemental material on the PNAS website.) Therefore, administration of the combination of an IMPDH inhibitor and a GPCR antagonist can be more effective than expected based on the individual use of each drug.

F. Rejection of claims 51-58

Claims 51-58 are rejected as obvious over Markovic *et al.*, US Patent No. 5,358,855 in view of Weber *et al.*, US Patent No. 5,405,837, and Chen *et al*, *Jap. J. Cancer* (1990).

Claims 51-58 are directed to methods to treat cancer by administering to a subject mizoribine or a related compound so that the plasma level of the compound is maintained between 0.5 and 50 micromolar for between 6 and 72 hours. Markovic *et al.*, Weber *et al.*, and Chen *et al*, are discussed above. The cited references do not provide all the elements of the claimed methods. Moreover, the specification at paged 26-37 discloses by prolonged administration of mizoribine or related compounds. Experimental support for the methods is found at, *e.g.*, Example 3, page 52.

The cited references, alone or together, do not teach or suggest all the elements of the claimed invention and do not provide a motivation for their combination to arrive at the claimed invention. The Office Action appears to rely on Weber *et al.* for many of the claim elements and for the alleged motivation to combine the references. According to the Office Action, Weber *et al.* teach administration of tiazofurin and ribavirin greater than 4,400 mg/m^2 or in a range between 1100-3300 mg/m^2. Weber *et al.* teach that the most effective dosage regime against cancer combines two IMPDH inhibitors: tiazofurin and ribavirin. Weber *et al.* teaches that daily infusion of tiazofurin can be toxic or can lead to resistance by the cancer. Column 2, lines 6-8. Weber *et al.* also teaches that ribavirin is less effective when administered alone. Column 1, lines 53-56. Therefore, Weber *et al.* provide no motivation to administer a single IMPDH inhibitor for treatment of cancer. At Example 3, page 52, Applicants provide experimental evidence that continual exposure of cancer cells to mizoribine for time periods between 24 and 72 hours decreases the IC_{50} of the drug up to ten fold. These surprising results support the non-obviousness of the claimed methods.

In view of the above arguments and remarks, withdrawal of the rejections for alleged obviousness is respectfully requested.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

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Activation of anti-hepatitis C virus responses via Toll-like receptor 7

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Contributed by Dennis A. Carson, December 15, 2005

IFN-α is used to suppress the replication of hepatitis C virus (HCV) in chronically infected patients with partial success. Here we present evidence showing that a ligand of Toll-like receptor 7 (TLR7) can induce anti-HCV immunity not only by IFN induction, but also through an IFN-independent mechanism. Human hepatocyte line Huh-7 carrying an HCV replicon expressed TLR7, and activation of the receptor induced several antiviral genes including IFN regulatory factor-7. Inhibitors of the enzyme inosine monophosphate dehydrogenase augmented both IFN-dependent and -independent antiviral effect. Prolonged exposure of Huh-7 cells to a TLR7 ligand [SM360320 (9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine)], alone or in combination with an inosine monophosphate dehydrogenase inhibitor, reduced HCV levels dose dependently. Immunohistochemical analysis of livers shows that TLR7 is expressed in hepatocytes of normal or HCV-infected people. Because TLR7 agonists can impede HCV infection both via type I IFN and independently of IFN, they may be considered as an alternative treatment of chronic HCV infection, especially in IFN-α-resistant patients.

inosine monophosphate dehydrogenase | IFN regulatory factor

More than 170 million people, ≈3% of the world's population, are infected with hepatitis C virus (HCV) and 55–85% of patients become chronically infected (1, 2). Many develop chronic liver disease, leading to cirrhosis and hepatocellular carcinoma. Combination therapy with polyethylene glycol modified IFN-α and ribavirin suppresses HCV replication in 40–80% of patients (3–5). However, severe side effects are associated with this treatment, leading to poor patient compliance. For these reasons, it is crucial to develop alternative therapies (6). These might include agents that selectively stimulate the production of IFN-α, and drugs that potentiate its anti-HCV effects.

The type I IFNs (IFN-α and IFN-β) are required for the efficient induction of a T helper 1 (Th1) immune response in humans (7). The activation of certain Toll-like receptors (TLRs), particularly TLR7 and TLR9, induces the production of type I IFNs, and thus primes the host for a Th1 adaptive immune response. Various synthetic nucleoside analogs related to guanosine protect mice in several models of RNA viral infection by induction of type I IFN via TLR7 stimulation (8, 9).

Recent reports have uncovered the key molecules in the TLR-induced signaling pathways that lead to type I IFN induction (11). Induction of type I IFN via TLR7 or TLR9 depends on the adaptor molecule MyD88 (10). TANK Binding Kinase 1 and IκB kinase i/ε (IKKi/ε) are activators of IFN regulatory factor-3 (IRF-3) and IRF-7 during TLR-mediated type I IFN production (11–13). These IRF molecules are phosphorylated in the cytosol and are translocated to the nucleus upon activation (13). DNA-dependent protein kinase (DNA-PK) has also been shown recently to phosphorylate and activate IRF-3 (14, 15). Activity of IRF-3 (16) and IRF-7 expression (17) are inhibited in HCV replicon cells. The IRFs can both foster type I IFN production (18) and enhance the antiviral activity of these cytokines (19). Thus, pharmacologic agents that activate IRFs in hepatocytes may exert direct anti-HCV effects, as well as potentiate IFN action.

Pharmacologic inhibitors of the enzyme inosine monophosphate dehydrogenase (IMPDH), including ribavirin and mizoribine, have been reported to have activity against RNA viruses (18–20). A recent report suggested that ribavirin could induce IRF-7 in cells infected with respiratory syncitial virus (21). This finding implied that IMPDH inhibitors might augment the antiviral activity of drugs that similarly induce IRF signaling.

Here we show that a TLR7 ligand 9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine (SM360320), can inhibit HCV replication in hepatocytes via a type I IFN-independent mechanism in addition to its IFN-mediated activity. The antiviral activity is enhanced by IMPDH inhibition in both cases. TLR7 is detectable in cultured Huh-7 hepatocytes and in the livers of most HCV-infected patients. These results raise the possibility that high-affinity TLR7 stimulants may be able to control chronic HCV infection *in vivo*, both by induction of IFN and by direct activation of antiviral mechanisms in infected hepatocytes.

Results

TLR7 Is Expressed in HCV-Infected Hepatocytes. Flow cytometry revealed that several TLRs are expressed in Huh-7 hepatoma cells, albeit mostly in intracellular compartments (Fig. 1*A*). TLR7 expression was most prominent. Anti-TLR7 antibody specifically recognized human TLR7 stably expressed in HEK293 cells (Fig. 5, which is published as supporting information on the PNAS web site). TLR7 mRNA was also expressed in Huh-7 cells (Fig. 1*B*), consistent with the flow cytometry data. In addition, various levels of TLR7 were detectable by immunohistochemistry in hepatocytes of normal, HCV-infected, and carcinoma liver tissues (Fig. 1 *C* and *D*) but not detectable in fibroblasts of HCV-infected livers (Fig. 1*E*).

A Potent TLR7 Ligand Suppresses HCV Replication. We investigated whether activation of different TLRs could inhibit replication of HCV in the Huh-7 replicon system (20). Treatment with prototype pharmacologic activators of TLR2, TLR3, TLR4, TLR5, TLR7, and TLR9 did not reduce the level of HCV protein (NS5A) as measured by Western blotting (Fig. 2*A*). A TLR2 ligand, pam3cys, actually enhanced the level of NS5A (Fig. 2*A*). Various 8-hydroxyadenine analogs were reported recently to be

Conflict of interest statement: No conflicts declared.

Abbreviations: HCV, hepatitis C virus; TLR, Toll-like receptor; IKK, IκB kinase; IRF, IFN regulatory factor; DNA-PK, DNA-dependent protein kinase; SM360320, 9-benzyl-8-hydroxy-2-(2-methoxyethoxy)adenine; IMPDH, inosine monophosphate dehydrogenase; BMDM, bone marrow-derived macrophages.

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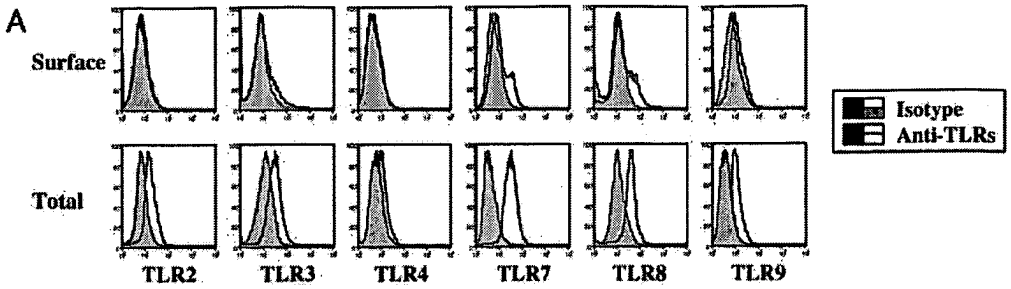
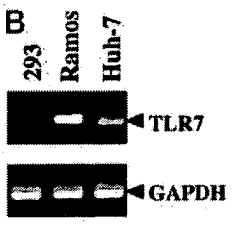
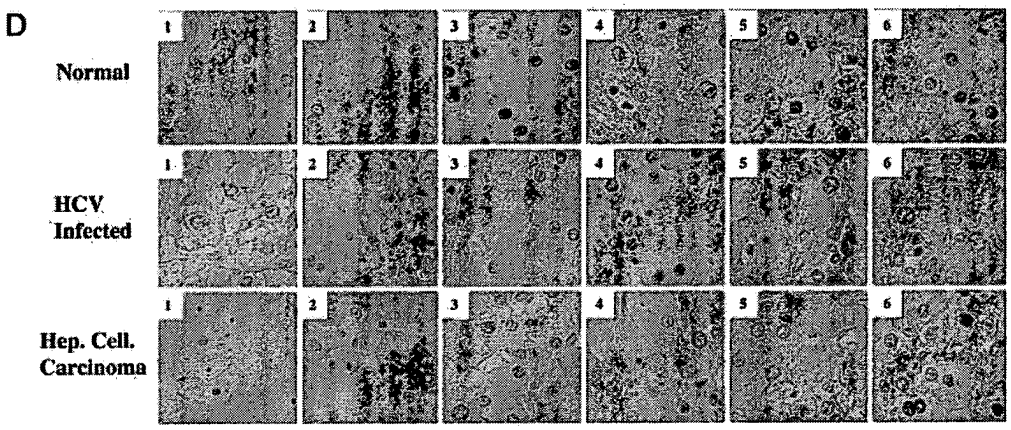
EXHIBIT A





C

Liver Tissues	TLR7 Expression Level (0 to 5)
Normal (n=11)	1, 0, 2, 2, 3, 4, 3, 4, 3, 5, 4
HCV-infected (n=11)	5, 4, 1, 2, 4, 0, 5, 5, 1, 1, 1
Hepatocellular carcinoma (n=14)	2, 1, 0, 0, 0, 5, 0, 5, 0, 0, 2, 1, 1, 0
Adenocarcinoma (n=2)	0, 4
Margin of Carcinoma (n=21)	5, 3, 5, 3, 5, 1, 3, 4, 1, 5, 1, 1, 2, 2, 2, 3, 0, 1, 2, 2, 1
Intrahepatic Cholangiocarcinoma (n=2)	0, 0,
Clear Cell Carcinoma (n=3)	0, 2, 1



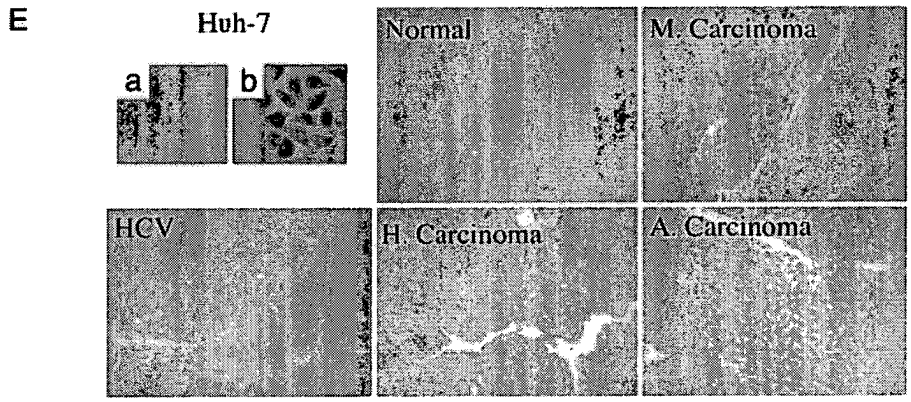


Fig. 1. Expression of TLR7 in hepatocytes. (*A*) Expression of TLR7 antigens on the surface and in the cytoplasm of Huh-7 replicon cells was assessed by antibody staining and FACS, as described in *Materials and Methods*. (*B*) Extracts of Huh-7 hepatocytes, HEK293 kidney cells (negative control), and Ramos B lymphocytes (positive control) were used to amplify TLR7 specific mRNA, as described in *Materials and Methods*. (*C* and *D*) Human liver tissue arrays were stained with TLR7 antibody after antigen recovery. The expression levels of TLR7 were estimated in different specimens using an arbitrary 0–5 scale. (*E*) Differential expression of TLR7 in human hepatocytes, compared to fibroblasts, was assessed by immunohistochemistry. A representative TLR7-stained tissue from each category is presented. M, margin of carcinoma; H, hepatocellular carcinoma; HCV, HCV-infected liver; A, adenocarcinoma.

potent inducers of type I IFNs (21). By genetic complementation in HEK293 cells, we identified SM360320 as a specific TLR7 ligand (Fig. 2*B*). Accordingly, SM360320 induced typical TLR7-mediated cytokines in human PBL (22), such as IFN-α, but little IL-6, IL-10, or IL-12, whereas R848, a ligand for TLR7 and TLR8 in humans, induced the latter cytokines in large quantities (Table 1, which is published as supporting information on the PNAS web site). However, in mouse splenocytes, where TLR8 is silent (23), SM360320 was at least 10 times more potent than R848 in induction of cytokines (Fig. 6, which is published as supporting information on the PNAS web site). Conditioned medium from SM360320- or R848-activated human PBL signif-

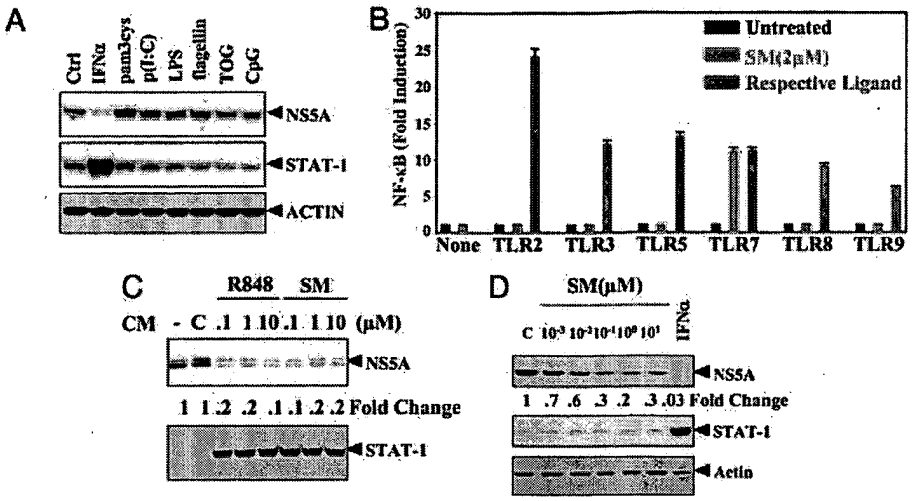


Fig. 2. Suppression of HCV replication by a TLR7 ligand, SM360320. (*A*) Huh-7-replicon cells were stimulated with the indicated TLR ligands or with IFN-α (1 ng/ml) for 24 h, and the level of the HCV NS5A protein or IFN-inducible phospho-STAT-1 was measured by Western blotting. [pam3Cys at 5 μg/ml, p(I:C) at 5 μg/ml, LPS at 100 ng/ml, flagellin at 10 ng/ml, TOG at 100 μM, and CpG at 5 μg/ml.] (*B*) HEK293 cells were transfected with vectors encoding the indicated TLRs, plus a NF-κB luciferase reporter, and then were stimulated with SM360320 (2 μM) or the indicated control ligands for 8 h. Luciferase activities were normalized to β-galactosidase activities in the same cells, as described (9). (*C*) Conditioned medium (CM) from PBL stimulated with the indicated drugs or untreated medium (C, 10 μl) was applied to Huh-7 cells carrying the HCV replicon. NS5A or STAT-1 levels were measured by Western blotting after 24 h. (*D*) Huh-7 cells were treated with the indicated concentrations of SM360320 for 24 h, and the levels of NS5A, STAT-1, or actin were measured by Western blotting.

icantly inhibited HCV replication (Fig. 2*C*), consistent with the high levels of type I IFN in the medium. However, SM360320 by itself inhibited HCV replication in Huh-7 cells in a dose-dependent manner (Fig. 2*D*). In contrast to the PBL-conditioned medium derived from SM360320-stimulated cells, the direct inhibition of HCV replication by SM360320 in Huh-7 cells was not mediated by extracellular type I IFN because (*i*) it was not inhibited by neutralizing anti-IFN-α receptor antibodies, and (*ii*) STAT-1 was not activated after drug treatment (Fig. 3*A*). However, the antiviral effect was TLR7 dependent, because chloroquine, an inhibitor of endosomal maturation (9), blocked the SM360320 anti-HCV activity (Fig. 3*B*). Treatment of Huh-7 cells with SM360320 or IFN-α induced several antiviral proteins, of which IRF-7 was prominent (Fig. 3*C*). However, NF-κB was activated only by SM360320 as expected, and MxA, an IFN-α target gene, was not induced by SM360320 (Fig. 3*C*). SM360320 reduced the level of HCV RNA in Huh-7 cells by >60% after overnight incubation, and by almost 80% after 2 weeks of treatment (Fig. 3*D*).

IMPDH Inhibitors Enhance the Anti-HCV Activity of TLR7 Ligands. Two IMPDH inhibitors, ribavirin and mizoribine base, were screened for inhibition of HCV replication, but had no activity at nontoxic concentrations (Fig. 7*A*, which is published as supporting information on the PNAS web site). However, the addition of an IMPDH inhibitor to SM360320 augmented the anti-HCV activity of the TLR7 ligand (Fig. 4*A*). Whereas IFN-α completely eliminated HCV RNA in 2 weeks, SM360320 treated cells still harbored 10–20% of HCV RNA at the end of the experiment (data not shown). To elucidate how IMPDH blockade might increase the consequences of TLR7 activation, we tested whether the enzyme inhibitors could stimulate and/or enhance signaling pathways induced by SM360320. The IMPDH inhibitors enhanced TLR7-induced activation of NF-κB in bone marrow-derived macrophages (BMDM) (Fig. 4*B*), increased the protein levels of IRF-1 and IRF-7 (Fig. 4*C*), and induced translocation of IRF-1 and IRF-3 to the nucleus (Fig. 7*B*). IRF-7 undergoes ubiquitination when activated by a TLR ligand (24) in addition to phosphorylation. Both TLR7 ligands and IMPDH inhibitors independently induced ubiquitination of IRF-7, which was further enhanced by the drugs in combination (Fig. 4*D*). The enzymes DNA-PK (14, 15) and IKKi/ε (11, 25) have been reported to play a role in the phosphorylation of IRFs (11, 13). Both TLR7 ligands and IMPDH inhibitors activated DNA-PK and IKKi/ε (Fig. 7 *C* and *D*).

Discussion

Synthetic activators of certain TLRs induce type I IFNs, which are known to inhibit HCV replication. Here, we tested whether TLR stimulation can also exert an IFN-independent antiviral effect, using the Huh-7 HCV replicon system (20). The results showed that a synthetic TLR7 activator, SM360320, reduced HCV mRNA and protein levels in isolated Huh-7 hepatocytes, whereas other activators of TLRs were ineffective. The anti-HCV action of SM360320 was associated with stimulation of antiviral genes such as IFN response factor-7 (IRF-7), but not with activation of the IFN-responsive STAT-1 transcription factor. Moreover, anti-IFN-α receptor antibodies did not neutralize the antiviral effect of the drug. Collectively, these results suggest that potent synthetic TLR7 ligands may inhibit HCV replication not only by stimulation of IFN production, but also by direct activation of antiviral mechanisms in hepatocytes.

Pharmacologic inhibitors of the enzyme IMPDH enhanced IRF activity in Huh-7 cells, and similarly potentiated the anti-HCV activity of TLR7 stimulants. The IMPDH inhibitors also increased TLR activation of mouse BMDM cells and human peripheral blood leukocytes. The effect was not compound specific, because it was observed with different inhibitors of IMPDH and was abrogated by replenishment of guanine nucleotides (data not shown). Nucleotide pool imbalances are known to induce DNA-PK, which can subsequently activate IRF-3 (14). IRF-3 and IRF-7 may cooperate to regulate IFN production and responses (26).

In humans, the expression of TLR7 (and TLR9) is mainly confined to plasmacytoid dendritic cells and B lymphocytes. Recently, low levels of TLR7 and/or TLR9 have been reported in other cell types, including hepatocytes, particularly in the setting of chronic inflammation (27). The level of certain TLRs is up-regulated at sites of inflammation (28), such as hepatocytes

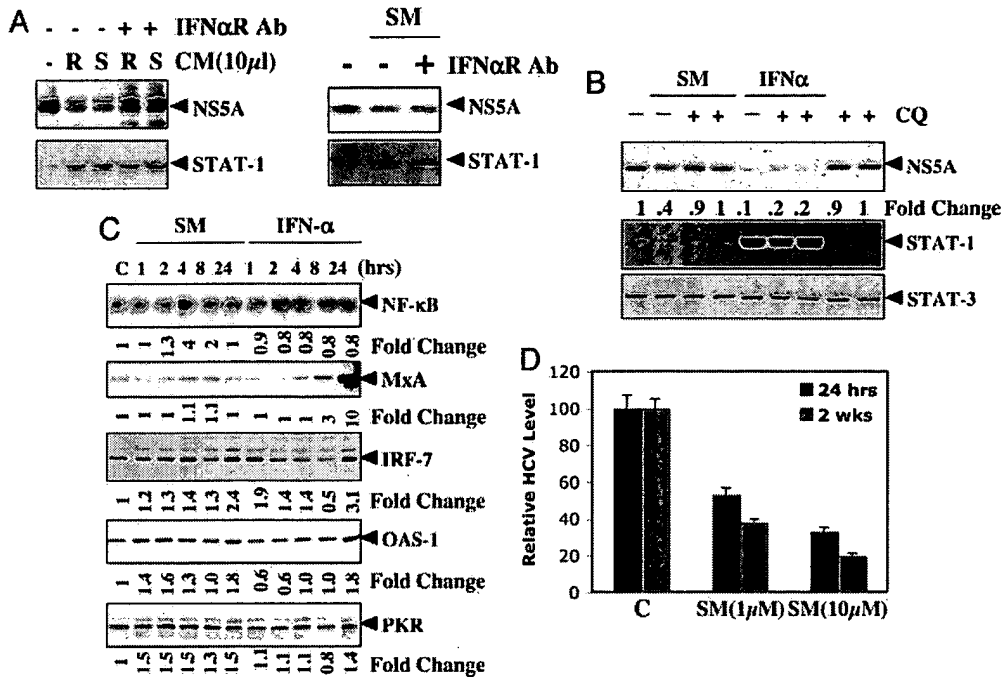


Fig. 3. Suppression of HCV replication by SM360320 through IFN dependent and independent pathways. (*A*) (*Left*) Conditioned medium (CM) was obtained from cells treated with R848 (R) or SM360320 (S), or unstimulated cells (C), and was applied to Huh-7 cells carrying HCV replicon. NS5A and phospho-STAT-1 levels were measured by Western blotting. Huh-7 cells carrying HCV replicon were pretreated with anti- IFN-α receptor (IFN-αR) antibody for 30 min followed by treatment with conditioned medium. (*Right*) Huh-7 cells were pretreated with neutralizing anti- IFN-αR antibody for 30 min followed by treatment with SM360320 (10 μM) or IFN-α (10 ng) for 24 h. The levels of NS5A and STAT1 were measured by Western blotting. The antibody blocked the IFN effect, but not the effect of SM360320. (*B*) Huh-7 cells were pretreated with the endosomal maturation inhibitor chloroquine (CQ, 10 μg/ml) for 30 min followed by addition of SM360320 (10 μM) or IFN-α (1 ng/ml). The levels of NS5A, STAT-1, or actin were measured by Western blotting after 24 h. (*C*) Huh-7 HCV replicon cells were treated with SM360320 (10 μM) or IFN-α (1 ng/ml) for the indicated time periods, and the levels of IRF-7, OAS-1, PKR, and MxA were measured by Western blotting. NF-κB actvation was measured by EMSA. (*D*) Huh-7 cells were treated with SM360320 (1 or 10 μM) for 24 h or 2 weeks, and the relative levels of HCV RNA were quantified by real-time PCR. For the 2-week assay, cells were treated every other day and were split every third day. The results are the average of two independent experiments.

(29). Expression pattern of TLR2 and TLR4 changes to become focal and irregular in HCV-infected hepatocytes (30). We detected TLR7 mRNA and protein in Huh-7 cells, and TLR7 antigen in normal and HCV-infected human liver. To determine whether the TLR7 was functional, Huh-7 cells were preincubated with chloroquine, which inhibits the maturation of endosomes, and hence the proper subcellular localization of TLR7 (9). Chloroquine blocked the anti-HCV effects of SM360320, without altering the antiviral activity of IFN-α. Thus, although one cannot rule out other off-target effects of SM360320, its anti-HCV activity requires endosomal TLR7.

The pattern of TLR7 expression in liver may explain why SM360320 exerted direct anti-HCV effects, whereas six other TLR activators did not, including R848 or 7-thia-8-oxoguanosine, a TLR7 ligand in clinical trials in HCV-infected patients (31). Although immunoreactive TLR7 was detectable in HCV-infected hepatocytes, the levels of the antigen were much lower than those found in infiltrating mononuclear cells in the same specimens (data not shown). In cells with low TLR7 concentrations, only a high-affinity ligand may be able to achieve maximal signal transduction. The mouse system, where TLR8 is not functional, allows a direct comparison of TLR7 ligands, and we found SM360320 was 10-fold more active than R848 (resiquimod) (Fig. 6), the most potent TLR7 stimulant studied to date (9, 23).

Hepatocytes normally express TLR3, which can be activated by the double-stranded RNA produced during HCV replication. Circumstantial evidence supporting a potential antiviral role of TLR activation in hepatocytes is the observation that HCV encodes a protease (NS3/4A) capable of cleaving the TLR3 adapter protein TRIF (32). However, TLR7 signaling does not require TRIF, but instead depends on the adapter protein MyD88, which is not specifically cleaved by the HCV protease. Accordingly, potent pharmacologic activators of TLR7 may be able to circumvent the HCV encoded protease that blocks TLR3.

In summary, our data provide evidence that TLR7-mediated immunity against HCV involves at least two different mechanisms: one depends on type I IFN production by leukocytes, and the other is mediated by TLR7 expressed by virally infected hepatocytes. Suppression of HCV replication via either mechanism is augmented by IMPDH inhibitors that prime the target cell through activation of one or more IRFs. Therefore, the combination of a TLR7 ligand and an IMPDH inhibitor may provide an orally active approach to HCV therapy.

Materials and Methods

Reagents. Deoxy-Guanosine (dG), deoxy-Cytosine (dC), deoxy-Adenosine (dA), and deoxy-Thymidine (dT) and mizoribine were purchased from Sigma. 7-Thia-8-oxo-G (TOG) was synthesized as described (33). R848 [also called resiquimod, 4-amino-2-ethoxymethyl-α,α-dimethyl-1*H*-imidazo(4.5-c)quinoline-1-ethanol] was from GL Synthesis (Worchester, MA). Ribavirin was a gift from ICN. Mizoribine base (5-hydroxy-1*H*-imidazole-4-carboxamide) was synthesized according to U.S. Patent no. 4,503,235 (P. D. Cook, 1985). Mizoribine base was used for most of the studies, because it was found to be equivalent to mizoribine in all biological systems, but was easier and less costly to prepare. SM360320 was synthesized as described (34). L-

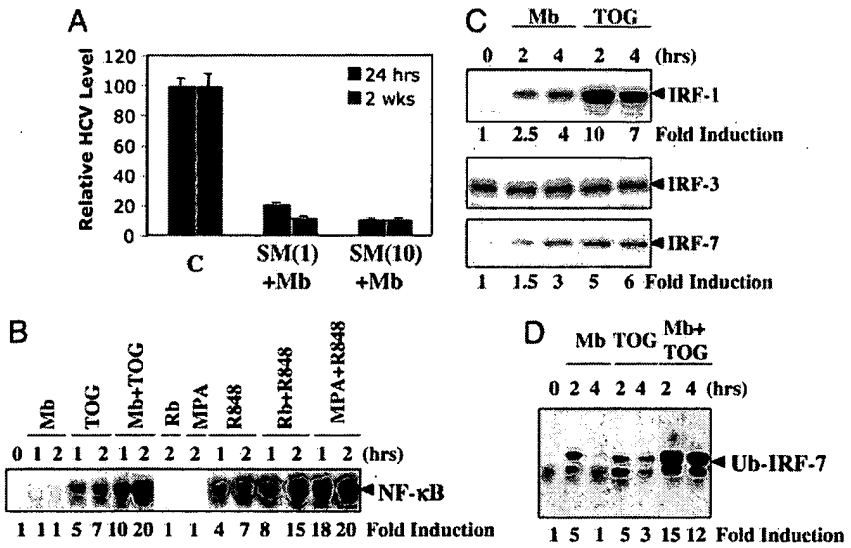


Fig. 4. Enhancement of TLR7-mediated anti-HCV activity by an IMPDH inhibitor. (*A*) Huh-7 cells were treated with SM360320 (1 or 10 μM), in some cases supplemented with mizoribine base (Mb, 1 μM) for 24 h or 2 weeks. The relative levels of HCV RNA were quantified by real-time PCR. The results are the average of two independent experiments. (*B*) BMDM were stimulated for the indicated time periods with the TLR7 ligand TOG (100 μM) or the TLR7/8 ligand R848 (1 μM) in the absence or presence of an IMPDH inhibitor [mizoribine (Mb, 10 μM), mycophenolic acid (MPA, 1 μM), or ribavirin (Rb, 100 μM)], and NF-κB activation was measured by EMSA. (*C*) BMDM were stimulated with Mb (10 μM) or TOG (100 μM) for the indicated time periods, and the levels of IRF-1, -3, and -7 were measured by Western blotting. (*D*) IMPDH inhibition enhances IRF-7 ubiquitination. BMDM were stimulated as indicated, and the cytosolic extracts were subjected to immunoprecipitation with anti-ubiquitin antibody, followed by SDS/PAGE and immunoblotting with anti-IRF-7 antibody.

Alanosine was obtained from the National Cancer Institute (NSC 153353). The source of the other TLR ligands has been described (9). HEK293 cells stably expressing human TLR7 were purchased from InvivoGen (San Diego, CA).

Antibodies. The following antibodies were used; antibodies to IKKβ, IKKi/ε, IRF-1, IRF-3, IRF-7, PKR, and JNK1 (Santa Cruz Biotechnology); antibodies to STAT-1, pSTAT-1, pERK, and *p*-p38 (Cell Signaling Technologies, Beverly, MA); anti-NS5A (Maine Biotechnology Services, Portland, ME); anti-DNA-PK (NeoMarkers, Fremont, CA); and anti-β-actin antibody (Sigma). Anti-OAS-1 antibody was purchased from Abgent (San Diego, CA). Anti-human TLR2, TLR3, and TLR4 antibodies were purchased from eBiosciences (San Diego, CA), and TLR7, TLR8, and TLR9 antibodies were purchased from Imgenex (San Diego, CA). Anti-human IFN-α receptor β-chain neutralizing antibody (clone MMHAR-2) was purchased from Research Diagnostics. Anti-MxA antibody was obtained from Othmar G. Engelhardt (Oxford University, Oxford).

Mice. C57BL/6 female mice, 6–8 weeks old, were purchased from The Jackson Laboratory. All experimental procedures were conducted in accordance with institutional guidelines for animal care and use. Murine splenocytes were isolated from the mice and cultured in RPMI medium 1640 supplemented with 10% FBS, as described (9). BMDM were isolated and cultured from the long bones of C57BL/6 mice for 7 days in L929 cell-conditioned medium (35).

Signaling Assays. Preparation of nuclear extracts and activation of NF-κB was measured by EMSA as described (36). Levels of different IRFs in the nuclei were measured before and after stimulation by using nuclear extracts of BMDM. IRFs were detected by Western blotting using specific anti-IRF-1, -IRF-3, and -IRF-7 antibodies. Activation of ERK, STAT-1, and p38 mitogen-activated protein kinase was assayed with antibodies specific to respective phosphorylated proteins. β-Actin levels were used for normalization of protein loading. The kinase activities of JNK (Jun N-terminal kinase), IKKβ (IκB kinase β), IKKi/ε (IκB kinase i/ε), and DNA-PK (DNA-dependent kinase catalytic subunit) were measured by an *in vitro* kinase assay using the respective recombinant proteins GST-cJun, GST-IκBα, and GST-p53 as substrates (36). Each enzyme was immunoprecipitated, and immune complexes were incubated with substrate and [γ-^{32}P]ATP for 30 min. The reaction samples were then electrophoresed (SDS/PAGE) and analyzed by autoradiography.

Activation of Human Peripheral Blood Leukocytes (PBL). Human blood samples were obtained from the San Diego Blood Bank. PBL were isolated from the blood samples by using Ficoll-Plaque Plus (Amersham Pharmacia), as described (9). PBL were washed twice with 50 ml of RPMI medium 1640, resuspended in 10 ml of RPMI medium 1640 with 10% FBS, and stimulated for 24 h with the indicated drugs. The cytokines in the supernatants were measured by using the Luminex multiple ELISA (Austin, TX), according to the manufacturer's instructions.

Studies on Huh-7 Cells with HCV Subgenome. Huh-7 cells, which contain an HCV replicon, were established and maintained as described (20, 37, 38). HCV-specific RNA levels in cell extracts were determined by real-time RT-PCR amplification with primers specific for the HCV untranslated region: 5′-GAG TGT CGT GCA GCC TCC AG-3′ (sense, 10 μM), 5′-CACTCGCAAG-CACCCTATCA-3′ (antisense, 10 μM), and 5′-FAM (carboxyfluorescein) CCCGCAAGACTGCTAGCCGAGTAGT-GTGG-TAMRA-3′ (probe, 10 μM; Biosearch, Novato, CA). RT reaction mixtures were incubated for 50 min at 60°C, followed by inactivation of the reverse transcriptase coupled with activation of Taq polymerase for 5 min at 95°C. Forty cycles of PCR were performed with cycling conditions of 15 s at 94°C, 10 s at 55°C, and 1 min at 69°C. The real-time PCR signals were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Applied Biosystems). To investigate the effect of SM36020 and mizoribine base on HCV replication for 2 weeks, Huh-7 cells carrying replicon were treated with the indicated drugs every 2 days and cells were split every 3 days. HCV

replication was also assessed by Western blotting using anti-NS5A antibody, and the relative changes in the level of NS5A or other proteins were measured with the software IMAGEJ. To assess TLR expression, the cells were detached with 20 mmol/liter EDTA in PBS. Cells were fixed and permeabilized by using CytoFix/CytoPerm (BD Biosciences). Cells were incubated in PBS containing 2% BSA for 30 min on ice and incubated for 1 h with either anti-hTLRs or isotype control at 1:200 dilution. For TLR7, TLR8, and TLR9, cells were washed in PBS/BSA twice and incubated with anti-rabbit IgG-FITC for TLR7 and TLR8 and anti-mouse IgG1-PE (BD PharMingen) for TLR9 for 30 min on ice. Antibody binding was detected by using a FACSCalibur (BD PharMingen). The following primers were used to amplify TLR7 message in human cell lines: Forward primer 5′-AGT GTC TAA AGA ACC TGG-3′ and reverse primer 5′-CTT GGC CTT ACA GAA ATG-3′. The annealing temperature was 55°C.

Immunohistochemistry of Human Liver Tissues. Pathologically confirmed human liver tissue arrays were obtained from U.S. Biomax (Rockville, MD). Deparafinization, inactivation of endogenous peroxidases, and antigen recovery were performed as described (39). Tissues were incubated with polyclonal anti-TLR7 antibody (1:25) overnight at 4°C, followed by anti-rabbit IgG-biotin (1:100) for 30 min and streptavidin–HRP (1:500) for 30 min at room temperature. The samples were developed with AEC kit (Vector Laboratories) and counterstained with Mayer's hematoxylin. The slides were graded for the intensity of hepatocytes staining by an arbitrary 0–5 scale.

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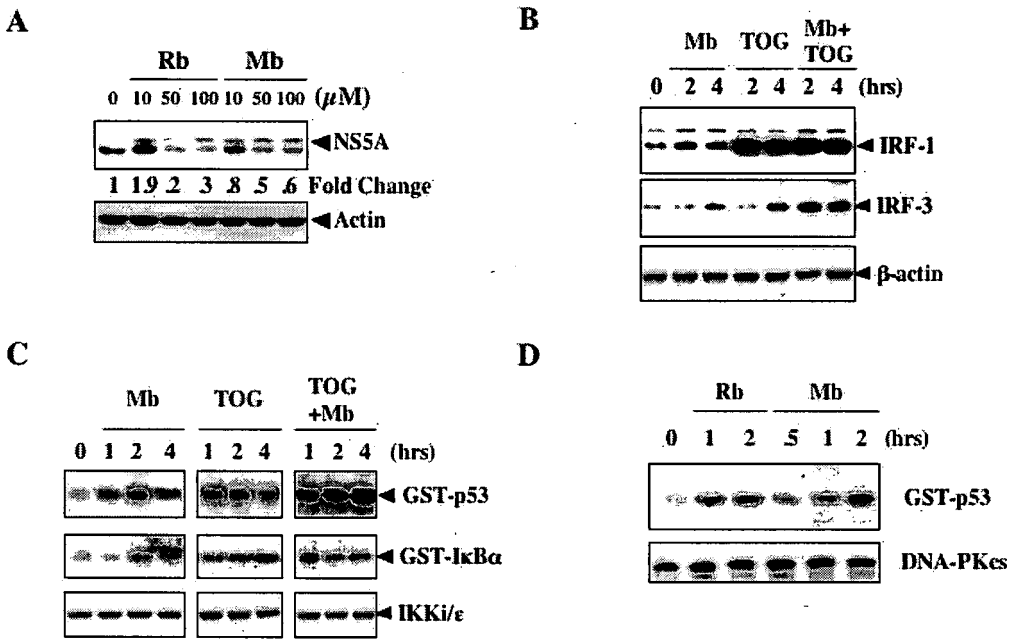

Fig. 7

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Supporting Information

Files in this Data Supplement:

Supporting Figure 5

Fig. 5. Anti-TLR7 antibody is specific. The specificity of anti-TLR7 antibody was tested in 293 cells or 293 cells stably transfected with human TLR7 (InvivoGen, San Diego, CA). Cells were fixed and permeabilized with CytoFix/CytoPerm (BD Biosciences), stained with anti-TLR7 antibody followed by anti-rabbit antibody labeled with Alexa Fluor 488, and analyzed by FACS.

Supporting Figure 6

Fig. 6. SM is a more potent TLR7 activator than R848. Splenocytes from B6 mice were stimulated with the indicated concentrations of SM or R848 for 24 h, and IL-6 level was measured by ELISA. Similar results were obtained for IL-12 (data not shown).

Supporting Figure 7

Fig. 7. Inosine monophosphate dehydrogenase (**IMPDH**) inhibitors enhance TLR7-mediated signaling pathways and activate a distinct set of signaling pathways. (*A*) **IMPDH** inhibitors inhibit hepatitis C virus (HCV) replication at high concentrations. Replicon cells were treated with the indicated dose of mizoribine base (Mb) or ribavirin (Rb) for 24 h, and the levels of NS5A or actin were measured by Western blotting. (*B*) Mb activates IRF-1 and -3 and enhances TLR-7 mediated activation of IRF-1 and -3. Bone marrow-derived macrophages BMDM were stimulated as indicated and activation of IRF-1 and -3 (translocation to the nucleus) was measured by Western blotting. (*C*) Mizoribine base activates and enhances TLR-7 mediated activation of DNA-PKcs and IKKi/ε. BMDM were stimulated for the indicated time periods with Mb (10 μM), TOG (100 μM), or TOG plus Mb (10 μM), and activation of DNA-dependent protein kinase (DNA-PK) and IκB kinase i/ε (IKKi/ε) was measured by *in vitro* kinase assay. (*D*) **IMPDH** inhibitors activate DNA-PK. BMDM were stimulated for the indicated time periods with Mb (10 μM) or Rb (50 μM) and activation of DNA-PK was measured by *in vitro* kinase assay.

Supporting Table 1

Table 1. Human PBL from two different donors (1 × 107 cells per ml) were stimulated with SM or R848 at the indicated dose for 24 hrs and the level of cytokines was measured by Luminex assay according to the manufacturer's instruction. The average of each cytokine is shown. Samples were diluted 1:2 in serum diluent and incubated with appropriate antibody-conjugated beads (Biosource International) overnight at 4° C on Millipore Multiscreen plates (MABVN1210). Samples were eluted by using a vacuum manifold and secondary-biotinylated antibodies were added for 90 minutes to the plate. Streptaviden-PE was added for 30 minutes followed by several wash steps. Read-out was performed on a Luminex-100